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Yeast Populations Residing on Healthy or *Botrytis*-Infected Grapes from a Vineyard in Attica, Greece[▽]

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The yeast flora associated with healthy and *Botrytis*-infected grapes was assessed. Molecular identification methods assigned isolates to six genera and nine species. For the first time *Hanseniaspora opuntiae* was encountered as an inhabitant of the grape ecosystem. By using DraI, an informative restriction fragment length polymorphism pattern was generated to distinguish *H. opuntiae* from the closely related organism *Hanseniaspora guilliermondii*. *Botrytis* infection resulted in a larger population and greater diversity of yeasts enriched with fermentative or spoilage species.

Grape berries, especially the interface between soluble nutrients and the septic world, are common niches for yeasts. From a biotechnological point of view, grapes are the primordial source of the microorganisms necessary for alcoholic fermentation to occur, providing must with both beneficial and potentially spoilage species. Nevertheless, the yeast flora of grapes is surprisingly poorly documented (13, 19). As determined so far, the physiognomy of the grape microflora may change in response to factors such as the climate, grape variety, and geographical region (4, 18, 21). Biological invasion is a critical concern for widespread changes in the community. *Botrytis* is among the most important pathogens that cause grape damage (gray rot) or drying (noble rot), yet its role in yeast ecology has not been studied previously. We assessed and compared yeasts present on *Botrytis*-infected and healthy grapes. Different molecular methods were used for species identification, and the robustness of these methods is discussed below.

Grape samples were collected at the time of harvest (2005 vintage) from the experimental vineyard of the Agricultural University of Athens (37°58′N, 23°32′E; 30 m above sea level). The grapevines were treated with ground sulfur rock during the spring, and no other chemicals were applied after this. 'Mavroliatis' and 'Sefka', two red *Vitis vinifera* varieties, were included in the analysis. Vines of each variety were cultivated in parallel single rows that were 20 m long and 40 m apart. Healthy and rotten bunches of each variety were randomly and aseptically collected from throughout the rows. The plate-trapped antigen enzyme-linked immunosorbent assay was used to confirm *Botrytis* infection of rotten grapes, using monoclonal antibody BC-12.CA4 (14) as described previously (5). One hundred grams of randomly collected berries from each sample was aseptically crushed with a Stomacher (Lab Blender 400), and the pH of the juice was recorded. D-Glucose/D-fructose and ethanol contents were determined by using appropriate enzymatic kits (Boehringer Mannheim/R-Biopharm, Germany). Decimal dilutions (10^{-1} to 10^{-6}) in Ringer's solution were prepared, and 100-μl portions were spread on different culture media. For enumeration and isolation of total yeasts, non-*Saccharomyces* yeasts, *Saccharomyces*, and *Dekkera*/*Brettanomyces* spp., samples were spread in triplicate on Wallerstein laboratory nutrient agar (Oxoid Ltd.), lysine medium agar (Oxoid Ltd.), ethanol sulfite agar (11), and *Dekkera*/*Brettanomyces* differential medium (20), respectively, supplemented with 10 mg/liter biphenyl and 100 mg/liter chloramphenicol (Sigma). Twenty to 35 isolates were randomly selected from plates with 50 to 180 colonies and stored at −80°C until further analysis. Genomic DNA was isolated as described previously (1). The 5.8S internal transcribed spacer (ITS) rRNA region and the D1/D2 domain of the 26S rRNA gene were PCR amplified using the ITS1-ITS4 (24) and NL1-NL4 (12) primer pairs, respectively, as described previously (12, 16). For 5.8S ITS restriction fragment length polymorphism (RFLP) analysis restriction endonucleases HinfI, HaeIII, HhaI, and DraI (Taqara, Japan), as well as DdeI (New England Biolabs), were used. Fragments were separated by agarose (3%) electrophoresis and were detected by using ethidium bromide. PCR products of the 5.8S ITS or D1/D2 domain of one to six randomly selected isolates per distinct RFLP pattern were gel purified (QIAquick PCR purification kit; QIAGEN), and both DNA strands were directly sequenced (Macrogen; http://www.macrogen.com). BLAST searches were performed with the NCBI/GenBank database, and the ClustalX software (http://www-igbmc.u-strasbg.fr/BioInfo) was used to construct multiple-sequence alignments.

Botrytis infections in rotten samples were verified by enzyme-linked immunosorbent assays (data not shown). In sample M2, infection led to noble rot (13), accompanied by a great increase in the sugar content and a slight increase in the pH compared to the sugar content and pH of sample M1 (Table 1). In sample S2, gray rot was apparent, and the grapes were smaller and damaged; the sugar content and pH value were lower than those in sample S1. The concentration of ethanol was quite low in all samples (≤0.07 g/liter) except sample M2 (1.04 g/liter). Similar CFU counts were obtained on Wallerstein laboratory nutrient agar and lysine medium agar, whereas no colonies

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TABLE 1. Physicochemical characteristics and yeast counts for grape samples

Sample	Variety/status of berries	D-Glucose/D-fructose content (g/liter)	D-Glucose/D-fructose ratio	pH	Yeast concn (log_{10} CFU/g) on[a]:	
					WLNA	LA
M1	'Mavroliatis'/healthy	171.5	1.2	3.6	3.3 (0.66)	3.2 (0.64)
M2	'Mavroliatis'/noble rot	282.5	1.0	4.0	6.8 (0.80)	6.7 (0.24)
S1	'Sefka'/healthy	178.6	0.7	3.8	4.3 (0.42)	4.1 (0.56)
S2	'Sefka'/gray rot	126.5	1.1	3.6	5.7 (0.55)	5.6 (0.78)

[a] The yeast concentrations are the means for triplicate samples, and the values in parentheses are standard deviations. WLNA, Wallerstein laboratory nutrient agar; LA, lysine medium agar.

appeared on ethanol sulfite agar and *Dekkera*/*Brettanomyces* differential medium, suggesting that *Saccharomyces cerevisiae* and *Dekkera*/*Brettanomyces* spp., respectively, either were not present or were present at low levels. The size of the yeast population in grapes with noble rot (sample M2) was 10^3-fold greater than the size of the yeast population in sample M1, a fact that may be ascribed to the release of sugars on the skin (7). For berries with gray rot (sample S2) the corresponding difference was less remarkable (Table 1).

A total of 204 isolates were analyzed by performing a PCR-RFLP analysis of the 5.8S ITS rRNA (Fig. 1). By using HinfI, HaeIII, HhaI, or DdeI, eight different banding patterns were generated, which according to the data set of Esteve-Zarzoso et al. (6) corresponded to *Hanseniaspora uvarum*, *Hanseniaspora guilliermondii*, *Zygosaccharomyces bailii*, *Issatchenkia terricola*, *Issatchenkia occidentalis*, *Metschikowia pulcherrima*, *Aureobasidium pullulans*, and *Candida stellata*. Sequence analysis confirmed the presence and positions of experimental restriction sites. The results of identification based on sequence relationships with previously described strains and phylogenetic analysis (data not shown) were in agreement with previous results, with the following two exceptions. A few *H. guilliermondii* isolates exhibited higher levels of homology to the recently described strain *Hanseniaspora opuntiae* CBS 8820 (2) than to *H. guilliermondii* CBS 95 (2 and 10 differences in 646 nucleotides, respectively), and all isolates identified as *C. stellata* exhibited 99.8% sequence similarity to *Candida zemplinina* (15), while the level of homology to *C. stellata* CBS 157 was only 88%. In view of the results described above, the sequences

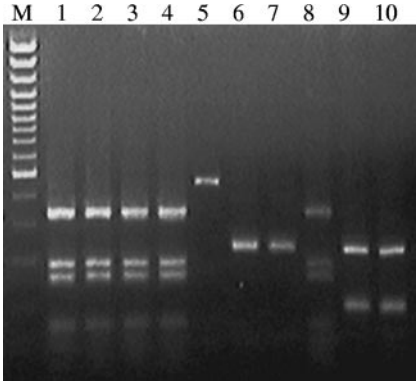

FIG. 1. Representative restriction patterns of the 5.8S ITS region of yeast isolates obtained with HinfI. Lane M, 100-bp molecular marker; lanes 1 and 2, *H. uvarum*; lanes 3, 4, and 8, *H. opuntiae*; lane 5, undigested PCR product from *C. zemplinina*; lanes 6 and 7, *C. zemplinina*; lanes 9 and 10, *I. terricola*.

of *H. opuntiae* and *H. guilliermondii* were inspected to identify informative RFLP patterns. In silico analysis and further experimental verification revealed that DraI generated distinct and readily distinguishable banding patterns for discrimination of these taxa based on two nucleotide differences in the ITS1 region (Table 2 and Fig. 2). After DraI digestion, 67% of the isolates were identified as *H. opuntiae* and 33% of the isolates were identified as *H. guilliermondii*. This enzyme was also used for analysis of *Candida* isolates, as previously suggested (23), to identify organisms as *C. zemplinina*. Thus, we suggest that DraI should be added to the list of enzymes previously proposed (6) for rapid discrimination of the novel species *H. opuntiae* and *C. zemplinina* from their close relatives *H. guilliermondii* and *C. stellata*, respectively.

Some heterogeneity among isolates of *M. pulcherrima* and *H. uvarum* was detected, suggesting that different strains of the same species may reside in such a restricted ecosystem. Two *M. pulcherrima* isolates from sample M2 exhibited a relatively high level of sequence divergence (3 of 321 nucleotides), and both of them were most closely related to *M. pulcherrima* accession number AY235809 (3). In the *H. uvarum* group, six isolates were divided into two clusters with 1% sequence divergence exhibiting 100% identity or two differences in 644 nucleotides compared to *H. uvarum* CBS 314 (2). In contrast, *C. zemplinina* and *I. terricola* isolates were clonal. In conclusion, PCR-RFLP analysis combined with a sequence analysis of the 5.8S ITS assigned isolates to six genera and nine species (Table 2). Identities were further corroborated by sequencing the D1/D2 domain. Genetic heterogeneity was observed in *M. pulcherrima* (2 of 484 nucleotides) and *H. uvarum* (1 of 547 nucleotides) isolates, which also showed relatively high 5.8S ITS divergence. No differences were detected between isolates and previously described strains except for MH509 and MH503, whose sequences differed at a single nucleotide from the sequences of *I. terricola* accession number U76345 and *H. uvarum* accession number U84229 (12), respectively.

Only two species, *H. uvarum* and *H. opuntiae*, were found in all four samples, and *H. uvarum* was the predominant organism (Fig. 3). This is the first report that *H. opuntiae* is a member of the grape ecosystem, probably because it was described only recently (2). The proportions of most other members of the communities were relatively low (<10%); the exception was *C. zemplinina* in samples S1 (22%) and S2 (12%). *C. zemplinina* was also previously found in *Botrytis*-infected musts (15, 22), and although this organism was found in both rotten samples, its high level in sample S1 suggests that it has a preference for a low glucose/fructose ratio in accordance with its strong fructophilic phenotype (15). *H. uvarum*, *H. guilliermondii*, *H. opun-*

TABLE 2. Sizes of the 5.8S ITS rRNA gene amplicons and the restriction fragments of the yeast isolates

Species	PCR product (bp)	Restriction fragments (bp)				
		HhaI	HaeIII	HinfI	DdeI	DraI
Z. bailii	770	310, 270, 95, 90	700, 70	330, 220, 160, 60		
H. uvarum	750	320, 310, 115	750	340, 190, 170, 60	290, 180, 90, 85, 75, 50	
H. guilliermondii	750	320, 310, 115	750	340, 190, 170, 60	360, 180, 85, 70, 50	420, 150, 130, 30
H. opuntiae	750	320, 310, 120	750	340, 190, 170, 60	360, 180, 85, 70, 50	420, 300, 30
A. pullulans	600	185, 180, 90, 90, 65	450, 150	290, 170, 140		
C. stellata	480	210, 105, 105, 60	480	240, 240		360, 120
C. zemplinina	480	210, 105, 105, 60	480	240, 240		310, 130, 40
I. occidentalis	480	240, 100, 80, 70	320, 100, 55	260, 110, 110		
I. terricola	460	120, 95, 95, 70, 60	290, 130	240, 110, 100		
M. pulcherrima	400	205, 100, 95	280, 100	200, 190		

tiae, and *I. terricola* were found on both healthy and infected 'Mavroliatis' grapes, while sample M2 also contained *Z. bailii*, *M. pulcherrima*, and *C. zemplinina*. For 'Sefka' grapes, *H. uvarum*, *H. opuntiae*, and *C. zemplinina* were present on both healthy and rotten grapes, while sample S2 also contained *I. occidentalis* and *I. terricola*. *A. pullulans* was the only oxidative yeast-like organism that was isolated solely from sample S1. The presence of *A. pullulans* on healthy grapes but not on diseased grapes is in accordance with previous findings (10, 17). *C. zemplinina* isolates were closely related to strain EJ1 (15) originating from California must, supporting the previous suggestion that this species may occur in geographically distant localities (22). *Z. bailii* was found on berries with noble rot. This yeast may reside in winery environments, where it is a real threat to product quality and preservation (9, 13). Therefore, its origins must be well established.

Infected grapes had higher yeast populations and more complex community structures as a result of the presence of new fermentative or potentially spoilage species. These observations are in accordance with previous suggestions concerning damaged grapes (8, 13). Interestingly, in a survey of Peloponnesus vineyards, it was found that *Botrytis*-infected grapes possessed a fermentative yeast community similar to the community described here, even though the healthy berries harbored a completely different community composed solely of oxidative species (A. A. Nisiotou and G. J. Nychas, unpublished data). *Botrytis* infection introduces structural changes in grapes that increase sugar accessibility and create new niches. In this context, resource availability may enhance population-level diversification and create opportunities for new species to become established. In addition, microbial communities are dynamic consortia of species populations, and therefore possible biological attributes of the system, such as interactive associations between *Botrytis* or other filamentous fungi often accompanying gray rot and yeasts, may not be excluded (8). Further

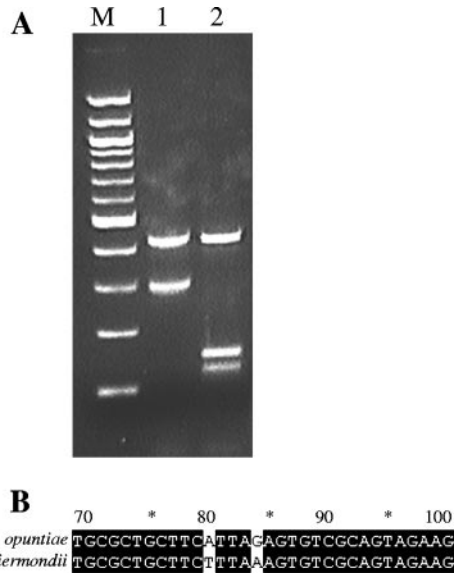

FIG. 2. (A) Informative restriction fragment length polymorphism in the 5.8S ITS amplicons of *H. opuntiae* and *H. guilliermondii* generated by DraI digestion. Lane M, molecular marker; lane 1, *H. opuntiae*; lane 2, *H. guilliermondii*. (B) Nucleotide sequence alignment of the 5.8S ITS rRNA region of *H. opuntiae* and *H. guilliermondii*, showing the informative DraI recognition site (5′-TTTAAA-3′) due to two nucleotide differences, as indicated by the white background.

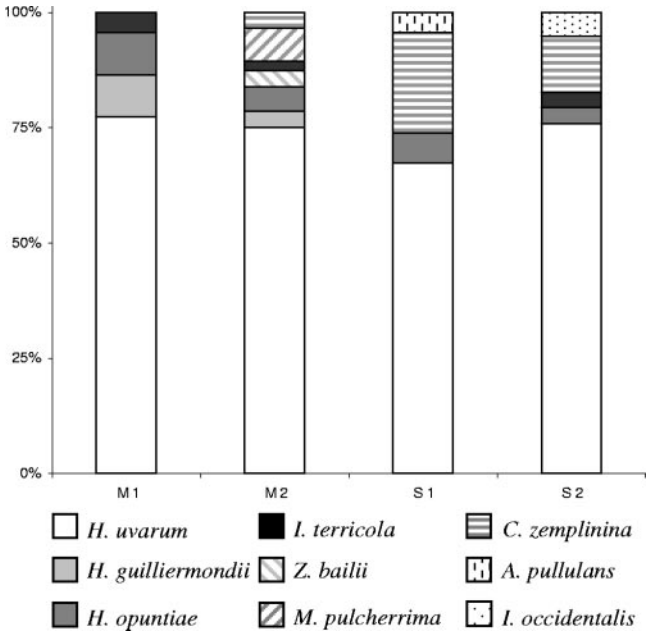

FIG. 3. Yeast species populations on healthy (samples M1 and S1) and *Botrytis*-infected (samples M2 and S2) grapes of cultivars Mavroliatis and Sefka.

research on grape microbial ecology should help address many unanswered questions concerning the impact of microbial consortia on the dynamic structure of yeast communities in grapes.

Nucleotide sequence accession numbers. Nucleotide sequences have been deposited in the GenBank under accession numbers DQ872854 to DQ872864 for the 5.8S ITS and DQ872865 to DQ872875 for the D1/D2 domain of isolates MH501, MH502, MB503, MH506, MB508, MH509, MB510, SB511, MB513, SH516, and SB517, respectively.

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